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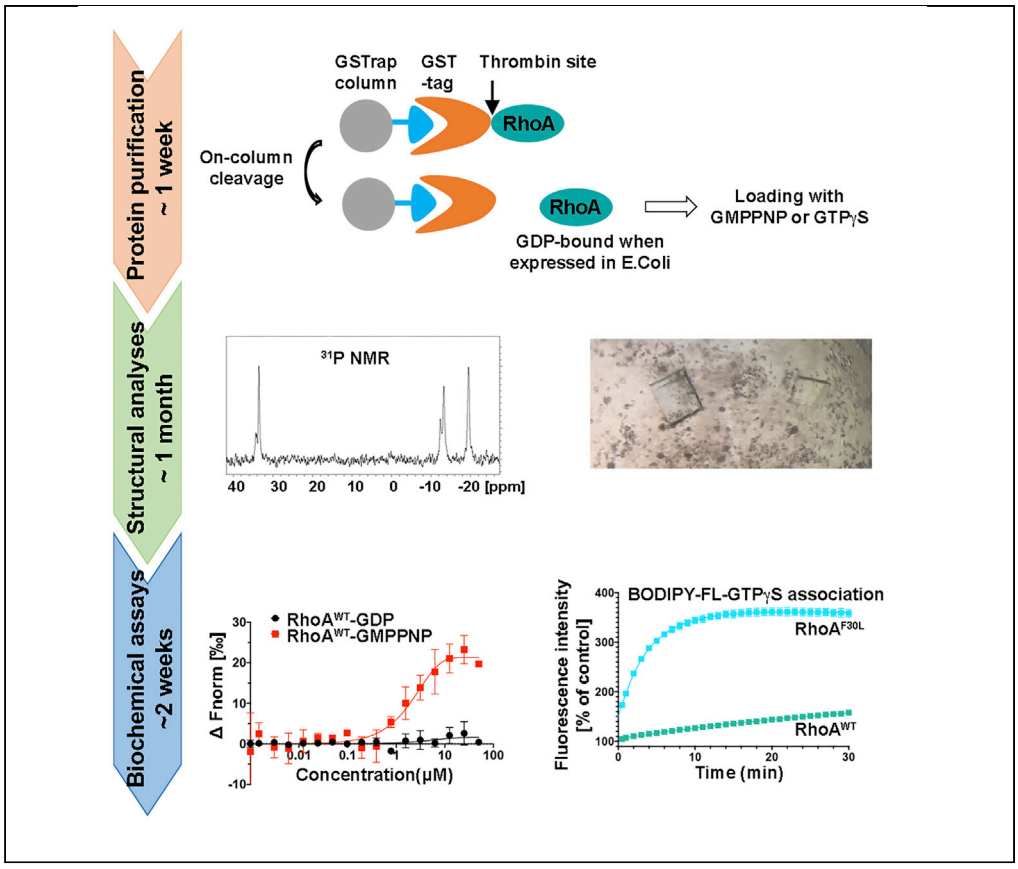
Protocol

Protocol for structural and biochemical analyses of RhoA GTPase



Ras GTPases in complex with GTP or GTP analog exhibit dynamic equilibrium between two interconvertible conformations—an inactive state 1 and an active state 2. Unlike Ras, it remains unclear if the GTP-bound form of Rho GTPases also exhibits multiple conformational states. Here, we describe a protocol for structural and biochemical analyses of RhoA GTPase. This protocol can be adapted for the characterization of other Rho GTPases.

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Highlights

Purification of wild-type and mutant RhoA in both GDP- and GTP analog-bound forms

Structural analyses of RhoA using both X-ray crystallography and NMR spectrometry

Biochemical assays of RhoA for effector binding and nucleotide exchange

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Protocol for structural and biochemical analyses of RhoA GTPase

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SUMMARY

Ras GTPases in complex with Guanosine triphosphate (GTP) or GTP analog exhibit dynamic equilibrium between two interconvertible conformations—an inactive state 1 and an active state 2. Unlike Ras, it remains unclear if the GTP-bound form of Rho GTPases also exhibits multiple conformational states. Here, we describe a protocol for structural and biochemical analyses of RhoA GTPase. This protocol can be adapted for the characterization of other Rho GTPases. For details on the use and execution of this protocol, please refer to Lin et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the detailed steps for structural and biochemical approaches for studying the Rho GTPase RhoA. We first describe the procedure for the expression and purification of RhoA in both GDP-bound and GTP analog-bound forms. We then describe the procedure of characterizing the conformational states of RhoA using ^{31}P NMR spectroscopy and X-ray crystallography. Lastly, we describe the classical biochemical assays for RhoA including effector pull-down, microscale thermophoresis (MST) binding assay, and nucleotide dissociation and association assays. The protocol may also be adapted to characterize other related small GTPases such as Rac1 and Cdc42.

Preparation for the expression plasmids for RhoA and mutants

Timing: 2 weeks

1. Obtain the expression plasmid encoding human full length RhoA in pGEX-2T vector from Jonathan Chernoff (Addgene Plasmid # 12202, https://www.addgene.org) (Bagrodia et al., 1995) and sequence with pGST 5′ and 3′ primers to confirm. This plasmid produces glutathione-S-transferase (GST) tagged RhoA with a thrombin site in between.
2. Design the following mutagenesis primers using QuikChange Primer Design server by Agilent (https://www.agilent.com/store/primerDesignProgram.jsp). The sequences of mutagenesis and pGST sequencing primers are listed in key resources table.

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Primer	Mutation
RhoA 181 truncation primer – forward and reverse	R182X (truncate at residue 181)
RhoA F25N primer – forward and reverse	F25N
RhoA G14V primer – forward and reverse	G14V
RhoA T37A primer – forward and reverse	T37A
RhoA F30L primer – forward and reverse	F30L

3. Obtain the following plasmids using QuikChange Lightning Site-Directed Mutagenesis Kit (For manufacturer's protocol: https://www.agilent.com/cs/library/usermanuals/Public/210518.pdf).

Plasmid	Protein after purification	Referred to in (Lin et al., 2021)
pGEX-2T-RhoA	RhoA full length	$RhoA^{FL}$
pGEX-2T-$RhoA^{1-181}$	RhoA (1-181)	$RhoA^{\Delta C}$
pGEX-2T-$RhoA^{1-181, F25N}$	RhoA (1-181, F25N)	$RhoA^{WT}$
pGEX-2T-$RhoA^{1-181, F25N, G14V}$	RhoA (1-181, F25N, G14V)	$RhoA^{G14V}$
pGEX-2T-$RhoA^{1-181, F25N, T37A}$	RhoA (1-181, F25N, T37A)	$RhoA^{T37A}$
pGEX-2T-$RhoA^{1-181, F25N, F30L}$	RhoA (1-181, F25N, F30L)	$RhoA^{F30L}$

a. For each mutagenesis step, prepare the reaction mixture as indicated below.

Reagent	Final concentration	Amount
10× reaction buffer	1× reaction buffer	5 μL
Template plasmid (10–100 ng/μL)	0.2–2 ng/μL	1 μL
Primer #1 (100 ng/μL)	2.5 ng/μL	1.25 μL
Primer #2 (100 ng/μL)	2.5 ng/μL	1.25 μL
DNTP mix	0.2 mM each	1 μL
QuikSolution reagent	Unknown	1.5 μL
Milli-Q water	n/a	38 μL
QuikChange Lightning Enzyme	0.05 U/μL	1 μL
Total	**n/a**	**50 μL**

Note: Milli-Q water should be added first and Quikchange Lightning Enzyme should be added last. DNTP mix is not stable and needs be thawed on ice right before use and put back to −20°C immediately afterwards. Quikchange Lightning Enzyme should be taken out of −20°C and kept on ice only when in use. Spin the reaction mix to get rid of any bubbles before PCR.

b. Perform the PCR reaction using the cycling parameters outlined below.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	20 s	18
Annealing	60°C	10 s	
Extension	68°C	2.5 min	
Final Extension	68°C	10 min	1
Hold	4°C	Forever	

Note: Extension time for the cycling is determined by the plasmid length, 30 s/kb. pGEX-2T-RhoA and RhoA mutant plasmids are ~5 kb, so use 2.5 min for the extension time.

c. *Dpn* I digestion of the amplification products

i. Add 2 μL of the *Dpn* I restriction enzyme directly to the amplification reaction.
ii. Gently mix by pipetting the solution up and down several times. Briefly spin down the reaction mixture and then immediately incubate at 37°C for 5 min.

d. Transformation of XL10-Gold ultracompetent cells.
i. Gently thaw the XL10-Gold cells on ice. For each reaction, aliquot 45 μL of the cells to a prechilled, autoclaved 1.5 mL Eppendorf tube.
ii. Add 2 μL of the β-ME mix in the kit to the 45 μL of cells. Swirl the tube gently, incubate on ice for 2 min.
iii. Transfer 2 μL of the *Dpn* I-treated DNA of each reaction to separate XL10-gold cells. Mix gently and incubate the reactions on ice for 30 min.
iv. Heat-shock the tubes at 42°C for 30 s, then incubate the tubes on ice for 2 min.
v. Add 0.5 mL SOC broth (without antibiotics) to each tube of bacteria, then incubate the tubes at 37°C for 1 h with shaking at 220 rpm. Pre-warm the LB-Ampicillin agar plates at 37°C at the same time.
vi. Plate the appropriate volume of each transformation reaction, usually 100–150 μL.
vii. Incubate the transformation plates at 37°C for 12–16 h.

e. Pick a colony with a sterile tip from each plate, inoculate into 5 mL LB-Ampicillin broth. Incubate at 37°C with shaking at 220 rpm for 12–16 h.
f. Extract the plasmids from the cells by a QiAprep Spin Miniprep Kit (QIAGEN) using the manufacturer's protocol. (https://www.qiagen.com/us/resources/resourcedetail?id=c1276626-d0b2-4e95-b2c3-dc81803a198c&lang=en)
g. Verify the construct by sequencing using pGEX-5′ and 3′ primers.

Preparation of LB agar plate

Timing: 2 h

4. Count 8 capsules of LB-agar and add 200 mL Milli-Q water. Autoclave at 121°C for 15 min.
5. Let agar cool down to 50°C–55°C (you should be able to touch the flask without burning). Add 200 μL Ampicillin stocks to make LB-Ampicillin plates.
6. Pour ~10–15 mL LB agar per 10 cm sterile Petri dish.
7. Let the plates cool down until solid. Seal the plates in plastic sleeve and store the plates at 4°C.

Preparation of stock solutions

Timing: 3 h

8. Antibiotics and IPTG socks.
   a. 1 M IPTG: dissolve 4.76 g IPTG in ~16 mL Milli-Q water, bring the final volume to 20 mL. Filter the solution with 0.22 μm syringe filter. Aliquot 1 mL/tube and store for up to 1 year at −20°C.
   b. 100 mg/mL Ampicillin: dissolve 2 g Ampicillin in ~16 mL Milli-Q water, bring the final volume to 20 mL. Filter the solution with 0.22 μm syringe filter. Aliquot 1 mL/tube and store for up to 1 year at −20°C.
9. Nucleotide stocks.
   a. 100 mM GDP: dissolve the 25 mg GDP in 564 μL Milli-Q water. Aliquot 50 μL/tube and store for up to 1 year at −20°C.
   b. 100 mM GMPPNP: dissolve the 25 mg GMPPNP in 425 μL Milli-Q water. Aliquot 50 μL/tube and store for up to 1 year at −20°C.
   c. 100 mM GTPγS: dissolve the 10 mg GTPγS in 178 μL Milli-Q water. Aliquot 50 μL/tube and store for up to 1 year at −20°C.
10. Stock buffers.
   a. 4 M NaCl: dissolve 233.76 g NaCl in ~800 mL Milli-Q water, bring the final volume to 1 L. Filter the solutions with 0.22 μm bottletop filter. Store for up to 3 years at 4°C.

b. 1 M Tris: pH 7.5 or pH 8.0. Dissolve 121.14 g Tris base in ~800 mL Milli-Q water, adjust the pH to 7.5 or 8.0 with 10 N HCl acid, bring the final volume to 1 L. Filter the solution with 0.22 μm bottletop filter. Store for up to 3 years at 4°C.
c. 1 M HEPES: pH 6.8 or pH 7.4. Dissolve 238.3 g HEPES (free acid) in ~800 mL Milli-Q water, adjust with solid NaOH first and then 10 M NaOH until the pH reaches 6.8 or 7.4, bring the final volume to 1 L. Filter the solution with 0.22 μm bottletop filter. Store for up to 3 years at 4°C.
d. 2 M $MgCl_2$: dissolve 19.0 g $MgCl_2$ in ~80 mL Milli-Q water, bring the final volume to 100 mL. Filter the solution with 0.22 μm bottletop filter. Store for up to 3 years at 4°C.
e. 1 M DTT: dissolve 3.1 g DTT in ~18 mL Milli-Q water, bring the final volume to 20 mL. Filter the solution with 0.22 μm syringe filter. Aliquot 1 mL/tube and store for up to 1 year at −20°C.
f. 3.5 M $(NH_4)_2SO_4$: dissolve 92.5 g $(NH_4)_2SO_4$ in ~160 mL Milli-Q water, bring the final volume to 200 mL. Filter the solution with 0.22 μm bottletop filter. Store for up to 3 years at 25°C.
g. 1 M $ZnCl_2$: dissolve 6.81 g $ZnCl_2$ in ~40 mL Milli-Q water, bring the final volume to 50 mL. Filter the solution with 0.22 μm syringe filter. Store for up to 3 years at 4°C.
h. 1 M $CaCl_2$: dissolve 5.55 g $CaCl_2$ in ~40 mL Milli-Q water, bring the final volume to 50 mL. Filter the solution with 0.22 μm syringe filter. Store for up to 3 years at 4°C.

Preparation of 15% sodium dodecyl sulfate (SDS) polyacrylamide gel

Timing: 1 h

11. Prepare the resolving gel (15%). Mix in the following order:

Reagant	Amount
H_2O	3.9 mL
ProtoGel (30%)	8.0 mL
ProtoGel Resovling Buffer (4×)	4 mL
TEMED	16 μL
Ammonium persulfate (APS), 10%	160 μL

Note: Add TEMED and APS to the SDS-PAGE resolving gel solution right before pouring the gel.

12. Pour the solution immediately to gel casting cassette, leaving ~2 cm below the bottom of the comb for the stacking gel. Make sure to remove bubbles.
13. Layer the top of the gel with isopropanol. This will help to remove bubbles at the top of the gel and will also keep the polymerized gel from drying out. In ~30 min, the gel should be completely polymerized.
14. Remove the isopropanol and wash out the remaining traces of isopropanol with Milli-Q water.
15. Prepare the stacking gel (4%). Mix in the following order:

Reagant	Amount
H_2O	3.05 mL
ProtoGel (30%)	0.65 mL
ProtoGel Stacking Buffer	1.25 mL
TEMED	5 μL
Ammonium persulfate (APS), 10%	25 μL

16. Pour stacking gel on top of the separation gel.

17. Add combs to make wells. In ~30 min, the stacking gel should become completely polymerized. Remove the combs and rinse the wells with Milli-Q water, the gel will be ready to use now.

Preparation of glutathione (GSH)-agarose beads

Timing: 2 days

18. Weigh out 1 g of lyophilized GSH-agarose beads. Soak in Milli-Q water for 12–16 h at 4°C in 50 mL conical tube. One-gram powder swells to > 10 mL gel.
19. Wash the beads with 5 volumes of Milli-Q water 3 times by spinning for 10 min at 6000 × *g*.
20. Store for up to 3 months at 4°C in equal volume of 2 M NaCl. The final beads are 50% (v/v) slurry.

KEY RESOURCES TABLE

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Antibodies		
RhoA Rabbit mAb (67B9)	Cell Signaling	Cat# 2117S
Bacterial and virus strains		
E. *coli BL21*(DE3) competent cells	New England Biolabs	Cat# C2527I
E. *coli* DH5α competent cells	New England Biolabs	Cat# C2987I
Chemical, peptides, and recombinant proteins		
RhoA full length	This paper	N/A
RhoA (1-181, F25N)	This paper	N/A
RhoA (1-181, F25N, G14V)	This paper	N/A
RhoA (1-181, F25N, T37A)	This paper	N/A
RhoA (1-181, F25N, F30L)	This paper	N/A
GST-Rhotekin-RBD (Rho binding domain)	This paper	N/A
Rhotekin-RBD	This paper	N/A
Thrombin from bovine plasma	Millipore/Sigma	Cat# T4648-10KU
50% w/v PEG 8000 solution	Hampton Research	Cat# HR2-535
100% 1,4-Dioxane	Hampton Research	Cat# HR2-617
GDP	Millipore/Sigma	Cat# G7127-25MG
GMPPNP	Millipore/Sigma	Cat# G0635-25MG
GTPγS	Millipore/Sigma	Cat# G8634-10MG
BODIPY-FL-GDP	Invitrogen	Cat# G22360
BODIPY-FL-GTPγS	Invitrogen	Cat# G22183
Ampicillin	GoldBio	Cat# A-301-25
IPTG	GoldBio	Cat# I2481C25
Lennox L Broth, granulated	RPI Research Products	Cat# L24066-5000.0
LB-agar medium, capsules	MP Biomedicals	Cat# 3002231
SOC medium	Millipore/Sigma	Cat# S1797-10X5mL
DTT	GoldBio	Cat# DTT10
Phenylmethanesulfonyl fluoride solution (~0.1 M in ethonal)	Millipore/Sigma	Cat# 93482-50mL-F
Lysozyme	VWR	Cat# VWR0663-10G
L-Glutathione reduced	Millipore/Sigma	Cat# G4251-5G
4× Laemmli Sample Buffer	Bio-Rad	Cat# 1610747
Precision Plus Protein Dual Color Standards	Bio-Rad	Cat# 1610374
ProtoGel (30%)	National Diagnostics	Cat# EC-890
ProtoGel Resolving Buffer (4×)	National Diagnostics	Cat# EC-892
ProtoGel Stacking Buffer	National Diagnostics	Cat# EC-893
TEMED	National Diagnostics	Cat# EC-503
Ammonium persulfate (APS)	National Diagnostics	Cat# EC-504
Albumin, bovine fraction V (BSA)	RPI Research Products	Cat# A30075-1000.0
Trans-Blot Turbo 5× Transfer Buffer	Bio-Rad	Cat# 10026938
Deuterium oxide	Acros Organics	Cat# 16630-1000

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REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
QiAprep Spin Miniprep Kit	QIAGEN	Cat# 27106
QuikChange Lightning Site-Directed Mutagenesis Kit	Agilent	Cat# 210518
PEG/Ion Screen	Hampton Research	Cat# HR2-126
Monolith Protein Labeling Kit RED-MALEIMIDE (Cysteine Reactive)	NanoTemper Technologies	Cat# MO-L004
Deposited data		
$RhoA^{WT}$-GDP	This paper	PDB: 6V6U
$RhoA^{WT}$-GMPPNP	This paper	PDB: 6V6M
$RhoA^{G14V}$-GDP	This paper	PDB: 6V6V
Oligonucleotides		
RhoA F25N primer – forward 5′-gggaactggtccttgctgttgactatgagcaagcatgt-3′	This paper	N/A
RhoA F25N primer – reverse 5′-acatgcttgctcatagtcaacagcaaggaccagttccc-3′	This paper	N/A
RhoA 181 truncation primer – forward 5′-ttttttcttcccacgtcaagcttgcagagcagctc-3′	This paper	N/A
RhoA 181 truncation primer – reverse 5′-gagctgctctgcaagcttgacgtgggaagaaaaaa-3′	This paper	N/A
RhoA G14V primer – forward 5′-atgtctttccacaggctacatcaccaacaatcacc-3′	This paper	N/A
RhoA G14V primer – reverse 5′-ggtgattgttggtgatgtagcctgtggaaagacat-3′	This paper	N/A
RhoA T37A primer – forward 5′-atagttctcaaacactgcgggcacatacacctctg-3′	This paper	N/A
RhoA T37A primer – reverse 5′-cagaggtgtatgtgcccgcagtgtttgagaactat-3′	This paper	N/A
RhoA F30L primer – forward 5′-gcacatacacctctggtaactggtccttgctgaag-3′	This paper	N/A
RhoA F30L primer – reverse 5′-cttcagcaaggaccagttaccagaggtgtatgtgc-3′	This paper	N/A
pGEX 5′ – forward sequencing primer 5′-gggctggcaagccacgtttggtg-3′	This paper	N/A
pGEX 3′ – reverse sequencing primer 5′- ccgggagctgcatgtgtcagagg-3′	This paper	N/A
Recombinant DNA		
pGEX-2T-RhoA	(Bagrodia et al., 1995)	Addgene Plasmid # 12202
pGEX-2T-$RhoA^{1\text{-}181,\ F25N}$	This paper	N/A
pGEX-2T-$RhoA^{1\text{-}181,\ F25N,\ G14V}$	This paper	N/A
pGEX-2T-$RhoA^{1\text{-}181,\ F25N,\ T37A}$	This paper	N/A
pGEX-2T-$RhoA^{1\text{-}181,\ F25N,\ F30L}$	This paper	N/A
pGEX-KG-Rhotekin-RBD	This paper	N/A
Software and algorithms		
Topsin™ ver 4.0.3	Bruker Corporation	https://www.bruker.com
Mosflm	(Battye et al., 2011)	https://www.ccp4.ac.uk/
Scala	(Evans, 2011)	https://www.ccp4.ac.uk/
Phenix	(Liebschner et al., 2019)	http://www.phenix-online.org/
Coot	(Emsley and Cowtan, 2004)	https://www.ccp4.ac.uk/
PyMOL	Schrödinger, LLC.	https://pymol.org/
Amber16	(Salomon-Ferrer et al., 2013)	https://ambermd.org/
Prism	GraphPad Software	https://www.graphpad.com/
Other		
Alkaline Phosphatase, Calf Intestinal (CIP) –Agarose	Millipore/Sigma	Cat# P0762-50UN
Glutathione (GSH)-Agarose	Millipore/Sigma	Cat# G4510-5ML

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REAGENT OR RESOURCE	SOURCE	IDENTIFIER
GSTtrap™ Fast Flow	Cytiva (GE Healthcare)	Cat# 17-5131-02
HiLoad™ 16/600 Superdex™ 200 pg	Cytiva (GE Healthcare)	Cat# 28-9893-35
4-15% Mini-PROTEAN TGX Stain-Free Protein Gels, 10 well, 50 µL	Bio-Rad	Cat# 4568084
Amicon® Ultra-15 Centrifugal Filter Unit (10 KDa MWCO)	Millipore/Sigma	Cat# UFC901024
Amicon® Ultra-15 Centrifugal Filter Unit (30 KDa MWCO)	Millipore/Sigma	Cat# UFC903024
Amicon® Ultra-0.5 Centrifugal Filter Unit (10 KDa MWCO)	Millipore/Sigma	Cat#: UFC501024
0.22 µm Syringe filter	VWR	Cat# 28145-477
Nalgene Bottletop Filters, 0.2 µm SFCA	Thermo Fisher Scientific	Cat# 290-3320
Pyrex Fernbach culture flasks 2800 mL	Millipore/Sigma	Cat# CLS44202XL
NMR tube	Bruker	Cat# Z112273
VDX plate with sealant	Hampton Research	Cat# HR3-171
22 mm Siliconized glass cover slides	Hampton Research	Cat# HR3-231
Immun-Blot® PVDF membrane	Bio-Rad	Cat#: 1620177
384-well Black/Clear Flat Bottom Polystyrene NBS Microplate	Corning	Cat# 3655
PCR plate, 96-well, non-skirted	Thermo Fisher Scientific	Cat# AB0600
Monolith NT.115 Series premium capillaries	NanoTemper Technologies	Cat# MO-K025
SI series Refrigerated Incubator Shaker	SHEL LAB	https://www.sheldonmanufacturing.com/shel-lab-incubators
Sonic Dismembrator Model 100	Fisher Scientific	Discontinued
Avanti J-26S XPI High-Performance Centrifuge	Beckman Coulter	Cat#: B14539
JLA-8.1000 Rotor	Beckman Coulter	Cat#: 363688
FiberLite F10BCI-6X500y Rotor	Thermo Fisher Scientific	Cat#: 096-062114
JA-30.50 Ti Rotor	Beckman Coulter	Cat#: 363420
Microcentrifuge 4515	Eppendorf	Discontinued
Peristaltic Pump P-1	Cytiva (GE Healthcare)	Cat#: 18111091
AKTApurifier FPLC	Cytiva (GE Healthcare)	Discontinued
Avance II 600 MHz NMR spectrometer	Bruker	Discontinued
Trans-Blot Turbo	Bio-Rad	Cat#: 1704150
Monolith NT.115	NanoTemper Technologies	https://nanotempertech.com/monolith/monolith-nt115/
EnVision Multimode Plate Reader	PerkinElmer	Cat#: 2105-0010

MATERIALS AND EQUIPMENT

Luria-Bertani (LB) media

Dissolve 20 g LB powder in 1 L Milli-Q water and autoclave the media at 121°C for 30 min. Store for up to 1 year at 25°C.

GST buffer A for GST-Rhotekin-RBD

GST buffer A for GST-Rhotekin-RBD contains 25 mM Tris pH 7.5, 1 M NaCl. Prepare 1 L and store for up to 1 year at 4°C.

Reagent	Final concentration	Amount
1 M Tris pH 7.5	25 mM	25 mL
NaCl	1 M	58.4 g
Milli-Q water	n/a	Up to 1 L

GST buffer A for GST-RhoA

GST buffer A for GST-RhoA contains 25 mM Tris pH 7.5, 1 M NaCl, 5 mM $MgCl_2$, 5 μM GDP. Prepare 1 L and store for up to 1 year at 4°C.

Reagent	Final concentration	Amount
1 M Tris pH 7.5	25 mM	25 mL
NaCl	1 M	58.4 g
2 M $MgCl_2$	5 mM	2.5 mL
100 mM GDP	5 μM	50 μL
Milli-Q water	n/a	Up to 1 L

GST elution buffer

GST elution buffer contains 50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM reduced glutathione. Freshly make 200 mL before use.

Reagent	Final concentration	Amount
1 M Tris pH 8.0	50 mM	10 mL
4 M NaCl	150 mM	7.5 mL
Reduced glutathione	10 mM	0.61 g
Milli-Q water	n/a	Up to 200 mL

Thrombin cleavage buffer

Thrombin cleavage buffer contains 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2.5 mM $CaCl_2$. Prepare 500 mL and store for up to 1 year at 25°C.

Reagent	Final concentration	Amount
1 M Tris pH 7.5	50 mM	25 mL
4 M NaCl	150 mM	18.75 mL
2 M $MgCl_2$	10 mM	2.5 mL
1 M $CaCl_2$	2.5 mM	1.25 mL
Milli-Q water	n/a	Up to 500 mL

Gel filtration buffer

Gel filtration buffer contains 20 mM Tris pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT. Prepare 500 mL freshly before use.

Reagent	Final concentration	Amount
1 M Tris pH 7.5	20 mM	10 mL
4 M NaCl	100 mM	12.5 mL
2 M $MgCl_2$	5 mM	1.25 mL
1 M DTT	1 mM	0.5 mL
Milli-Q water	n/a	Up to 500 mL

Nucleotide exchange buffer

Nucleotide exchange buffer contains 40 mM Tris pH 7.5, 200 mM $(NH_4)_2SO_4$, 10 μM $ZnCl_2$, 5 mM DTT. Prepare 20 mL, aliquot 1 mL/tube and store for up to 6 months at −20°C.

Reagent	Final concentration	Amount
1 M Tris pH 7.5	40 mM	0.8 mL
3.5 M $(NH_4)_2SO_4$	200 mM	1.14 mL
1 M $ZnCl_2$	10 μM	0.2 μL
1 M DTT	5 mM	100 μL
Milli-Q water	n/a	Up to 20 mL

CIP-agarose storage buffer

CIP-agarose storage buffer contains 2 M $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$. Prepare 50 mL and store for up to 1 year at 25°C.

Reagent	Final concentration	Amount
3.5 M $(NH_4)_2SO_4$	2 M	28.6 mL
2 M MgCl2	1 mM	25 μL
1 M $ZnCl_2$	0.1 mM	5 μL
Milli-Q water	n/a	Up to 50 mL

GST pull-down buffer

GST pull-down buffer contains 50 mM HEPES pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.3% Triton-X100, 1 mM DTT. Prepare 100 mL freshly before use.

Reagent	Final concentration	Amount
1 M HEPES pH 7.4	50 mM	5 mL
4 M NaCl	100 mM	2.5 mL
2 M $MgCl_2$	5 mM	0.25 mL
1 M DTT	1 mM	0.1 mL
Triton-X100	0.3%	0.3 mL
Milli-Q water	n/a	Up to 100 mL

MST sample storage buffer

MST sample storage buffer contains 20 mM HEPES pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 5 μM GDP or GMPPNP, 10% Glycerol. Prepare 50 mL and store for up to 1 month at 25°C.

Reagent	Final concentration	Amount
1 M HEPES pH 7.4	20 mM	1 mL
4 M NaCl	100 mM	1.25 mL
2 M $MgCl_2$	1 mM	25 μL
Glycerol	10%	5 mL
100 mM GDP/GMPPNP	5 μM	2.5 μL
Milli-Q water	n/a	Up to 50 mL

MST binding buffer

MST binding buffer contains 50 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween-20, 5 μM GDP or GMPPNP. Prepare 50 mL and store for up to 1 month at 25°C.

Reagent	Final concentration	Amount
1 M HEPES pH 7.4	50 mM	2.5 mL
4 M NaCl	150 mM	1.875 mL
2 M $MgCl_2$	10 mM	250 μL
Tween-20	0.05%	25 μL
100 mM GDP/GMPPNP	5 μM	2.5 μL
Milli-Q water	n/a	Up to 50 mL

BODIPY-FL-GDP loading buffer

BODIPY-FL-GDP loading buffer contains 20 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA. Prepare 100 mL and store for up to 1 year at 25°C.

Reagent	Final concentration	Amount
1 M Tris pH 7.5	20 mM	2 mL
4 M NaCl	100 mM	2.5 mL
0.5 M EDTA	1 mM	0.2 mL
Milli-Q water	n/a	Up to 100 mL

Nucleotide dissociation/association assay buffer

Nucleotide dissociation/association assay buffer contains 20 mM Tris pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$. Prepare 500 mL and store for up to 1 year at 25°C.

Reagent	Final concentration	Amount
1 M Tris pH 7.5	20 mM	10 mL
4 M NaCl	100 mM	12.5 mL
2 M $MgCl_2$	5 mM	1.25 mL
Milli-Q water	n/a	Up to 500 mL

STEP-BY-STEP METHOD DETAILS

Transformation and bacterial glycerol stock

Timing: 2 days

Transform the expression plasmids into *BL21*(DE3) and make bacterial glycerol stock for future use.

1. Transform the expression plasmids that encode $RhoA^{FL}$, $RhoA^{WT}$, $RhoA^{G14V}$, $RhoA^{T37A}$, $RhoA^{F30L}$, and Rhotekin-RBD into *BL21*(DE3) competent cells following the protocol describe above for transformation of XL10-Gold cells (No β-ME is needed for *BL21*(DE3) competent cells). Plate the transformed samples to LB-Ampicillin plates. Incubate at 37°C for 12–16 h.
2. Inoculate single colony into 5 mL LB media supplemented with 100 μg/mL Ampicillin. Incubate at 37°C with shaking at 220 rpm until OD_{600} reaches 0.5. Add 600 μL cell culture to 400 μL autoclaved 80% glycerol in a 2 mL screw-cap tube. Freeze the glycerol stock tube in liquid nitrogen and store at −80°C.

Expression and purification of $RhoA^{FL}$, $RhoA^{WT}$, $RhoA^{G14V}$, $RhoA^{T37A}$, and $RhoA^{F30L}$

Timing: 5 days for each sample

Prepare the RhoA samples for ^{31}P NMR spectroscopy, crystallization, and biochemical assays (Figure 1).

3. Inoculate with a gentle stubbing from the glycerol stock using a sterile pipette tip into 100 mL LB media supplemented with 100 μg/mL Ampicillin. Incubate at 37°C with shaking at 220 rpm for 12–16 h.

Note: If a reduced expression level is observed and the bacterial glycerol stock has been stored at −80°C for a long time, strike a new LB agar plate containing the appropriate antibiotics or re-transform *BL21*(DE3) to pick a single colony to start protein expression.

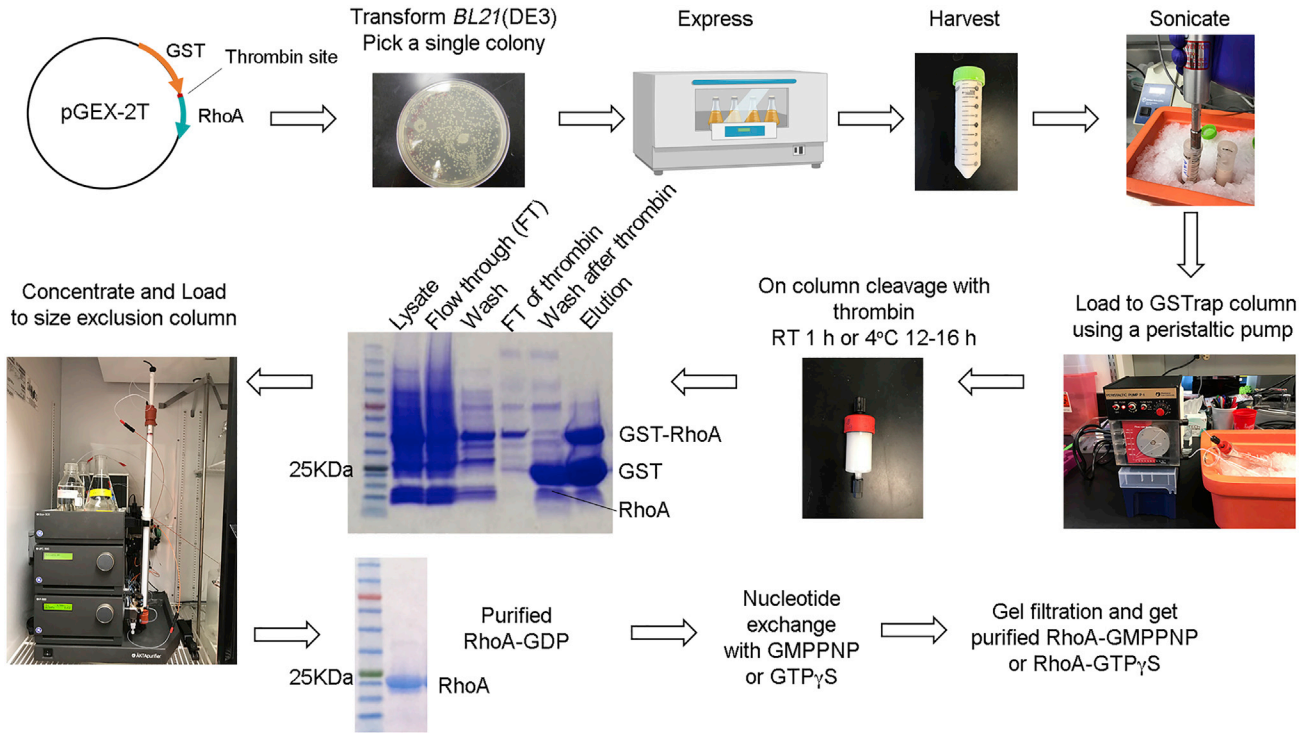

Figure 1. Procedure for the purification of RhoA and mutant proteins

The purification steps include GSTrap affinity column, on-column cleavage by thrombin, and size exclusion chromatography for GDP-bound RhoA proteins. GTP analog-bound WT or mutant RhoA protein is obtained after nucleotide exchange followed by size exclusion chromatography.

4. Dilute the fully-grown culture 1:100 in 6× 1 L LB media supplemented with 100 μg/mL Ampicillin in 6× 2.8-L Fernbach culture flasks. Incubate at 37°C with shaking at 220 rpm until OD_{600} reaches 0.6–0.8.
5. Add 0.2 mL 1 M IPTG to each flask to reach final concentration of 0.2 mM. Reduce the shaker temperature to 20°C, keep incubating with shaking for 12–16 h. Troubleshooting 1
6. Harvest cells by centrifuge at 4,000 × *g* for 20 min at 4°C and discard supernatant (add 20% v/v bleach to supernatant and sit for 20 min before dumping to sink)
7. Resuspend the pellet in 15 mL GST buffer A per liter cell culture by pipetting up and down using 10 mL transfer pipette. Transfer the re-suspended cells to 50 mL conical tubes.

   ⏸ **Pause point:** Add glycerol to re-suspended cells to ~10% (v/v), flash freeze in liquid nitrogen, store at −80°C for up to 6 months.

8. Add 1:100 PMSF (1 mM final concentration), 1:100 DTT (10 mM final concentration), sprinkler of lysozyme to the re-suspended cells.
9. Lyse the cells on ice by sonication for 5 min at 60% power with intervals of 20 s on and 10 s off.
10. Spin down the cell lysate at 35,000 × *g* for 1 h at 4°C to remove all debris and insoluble fractions.

    ⚠ CRITICAL: The Cell lysate should always be kept on ice during purification.

11. At the same time, wash a 5 mL GSTrap FF column with at least 5 column volume (CV) Milli-Q water and then 5 CV GST buffer A using a peristaltic pump at the speed of 5 mL/min.
12. Load the supernatant of cell lysate to the equilibrated GSTrap FF column at the speed of 1 mL/min.
13. Wash the column with at least 5 CV GST buffer A.

14. Wash the column with at least 2 CV Thrombin cleavage buffer.
15. Dissolve 10 mg thrombin in 2.5 mL Thrombin cleavage buffer, load to the column. Let the column sit at 25°C for 1 h or at 4°C for 12–16 h to cleave the proteins. Troubleshooting 2
16. Wash the column with 5 CV GST buffer A. Collect the flow through.

⚠ CRITICAL: Be careful not to let liquid run out and introduce air bubbles to the GSTrap column.

17. Concentrate the flow through to 2 mL final volume using Amicon Ultra-15 Centrifugal Filter Unit (10 KDa MWCO) at a speed of 6,000 × *g* at 4°C.
18. Inject the concentrated sample onto HiLoad 16/600 Superdex 200 pg column pre-equilibrated with 2 CV Gel filtration buffer and run at 1 mL/min. RhoA elutes at ~90 mL on this column.
19. Pool protein-containing fractions and concentrate the protein to ~1 mL using Amicon Ultra-15 Centrifugal Filter Unit (10 KDa MWCO) at a speed of 6,000 × *g* at 4°C.
20. Measure the concentration using Bradford reagent. Aliquot the sample, freeze the tubes in liquid nitrogen, then store the tubes at −80°C. The RhoA protein purified in this step is in the GDP-bound state.
21. Wash the CIP-agarose beads with 20 mM Tris pH 7.5 for 3 times, then add RhoA-GDP sample, 10 molar fold GMPPNP or GTPγS, bring the final volume to 1.5 mL using Nucleotide exchange buffer. Rotate at 4°C for 12–16 h.
22. Spin down the CIP-agarose beads by desktop centrifuge at 4°C for 2 min at maximum speed, transfer the supernatant to a new tube. Wash the beads with 0.5 mL 20 mM Tris pH 7.5, spin down, combine the wash with the supernatant. Inject the sample onto HiLoad 16/600 Superdex 200 pg column pre-equilibrated with 2 CV Gel filtration buffer and run at 1 mL/min. RhoA elutes at ~90 mL on this column. The CIP-agarose beads can be reused after being washed with 1 mL 20 mM Tris pH 7.5 for three times, and then stored in 1 mL CIP-agarose storage buffer at 4°C.
23. Pool protein-containing fractions and concentrate the protein to ~ 1 mL using Amicon Ultra-15 Centrifugal Filter Unit (10 KDa MWCO) at a speed of 6,000 × *g* at 4°C.
24. Measure the concentration using Bradford reagent. Aliquot the sample, freeze the tubes in liquid nitrogen, then store the tubes at −80°C. The resulted RhoA protein is in the GMPPNP- or GTPγS-bound state.

Note: For RhoA mutant G14V which has reduced intrinsic GTPase activity and impaired GAP-stimulated GTPase activity, the purified $RhoA^{G14V}$ at step 19 is a mixture of GDP-bound and GTP-bound protein. To remove GTP, extra 10 mM EDTA and 5 mM GDP can be added to the sample after step 16, and let the protein sit at 25°C for 3 h before proceeding to step 17.

Expression and purification of GST-Rhotekin-RBD

Timing: 5 days

Prepare the GST-Rhotekin-RBD protein for GST pull-down and for titration with RhoA for ^{31}P NMR data collection. This procedure is similar to RhoA purification.

25. Express GST-Rhotekin-RBD using steps 3–7 as above.
26. Purify GST-Rhotekin-RBD using steps 8–13 as above.
27. Elute the protein from the GSTrap column using 5 CV GST elution buffer.
28. Concentrate the elution to 2 mL final volume using Amicon Ultra-15 Centrifugal Filter Unit (30 KDa MWCO) at a speed of 6,000 × *g* at 4°C.
29. Inject the concentrated sample onto HiLoad 16/600 Superdex 200 pg column pre-equilibrated with 2 CV Gel filtration buffer and run at 1 mL/min. GST-Rhotekin-RBD elutes at ~60 mL on this column.

30. Pool protein-containing fractions and concentrate the protein to ~1 mL using Amicon Ultra-15 Centrifugal Filter Unit (30 KDa MWCO) at a speed of 6,000 × *g* at 4°C.
31. Measure the concentration using Bradford reagent. Aliquot the sample, freeze the tubes in liquid nitrogen, then store the tubes at −80°C.

^{31}P NMR spectroscopy

Timing: 3 days for each sample

Collect the ^{31}P spectra of RhoA and mutants in the GDP-bound, GMPPNP-bound, or GTPγS-bound states. GST-Rhotekin-RBD was titrated to the RhoA or RhoA mutant samples to identify the varying conformation states.

32. For ^{31}P NMR spectra collection, make sure the protein concentration > 0.4 mM. The RhoA proteins are in the Gel filtration buffer. Add 50 μL Deuterium oxide to 450 μL protein sample and transfer the sample to 5mm NMR tubes. Troubleshooting 3
33. Record 1D ^{31}P spectra at 275K and 298K on a Bruker Avance II 600 MHz spectrometer with 5 mm BBO Prodigy probe. Once the probe temperature is stable, insert the sample and allow the sample temperature to stabilize for 15 min minimum. At each temperature, adjust lock, tune and match on ^{31}P channel, and shimming before starting the data collection. Acquire the data using 1D pulse program "zgpg30" from Bruker pulse library with following parameters: time domain (TD) = 30k, DS = 8, spectral width (SW) = 100 ppm, acquisition time (AQ) = 0.6 s, relaxation delay (D1) = 3 s, and adjusted number of scans (NS) depending on the sample concentration.
34. Add increasing amount of GST-Rhotekin-RBD to the NMR sample (molar ratios of GST-Rhotekin-RBD : RhoA are 0.5, 1, and 2). After 15 min incubation at 25°C, the sample is concentrated back to 500 μL using Amicon Ultra-0.5 Centrifugal Filter Unit (10 KDa MWCO) at a speed of 6,000 × *g* at 4°C. Collect the ^{31}P spectra after each titration.
35. Process the spectra using the Topspin 4.0 software (Bruker). All spectra are manually phased, baseline corrected, and referenced to the phosphoric acid at 0.0 ppm.

Note: No extra GDP or GTP analogs should be added to the samples. The spectra of free GDP and GTP analogs should be collected in the same buffer as controls in parallel.

Crystallization and structure determination of RhoA and mutants

Timing: 2 weeks to 1 month

Crystallization and structure determination of the RhoA proteins in both GDP-bound and GMPPNP-bound states (Figure 2).

36. The crystallization screening is performed using commercially available PEG/ion Screen (Hampton research) and the crystallization conditions for RhoA structures deposited in the PDB (rcsb.org). PEG/ion screen is chosen as previous RhoA structures were all crystallized in conditions containing PEG. For example, RhoA^{G14V}-GTPγS (PDB: 1A2B) was crystallized in 20% PEG 8K, 15% 14-dioxane, 0.1 M Tris pH 8.5; RhoA-GDP (PDB: 1FTN) was crystallized in 28% PEG 8K, 0.1 M Na acetate pH 5.7, 0.5 mM Na citrate (Ihara et al., 1998; Wei et al., 1997).

⚠ CRITICAL: The Switch I & II regions of RhoA are highly flexible and may adapt a large ensemble of conformations. Crystallization conditions and the resulted crystal packing seem to have a role in selecting a particular conformation of the Switch regions. Therefore, it is important to take into account the effect of crystal packing when analyzing the structures.

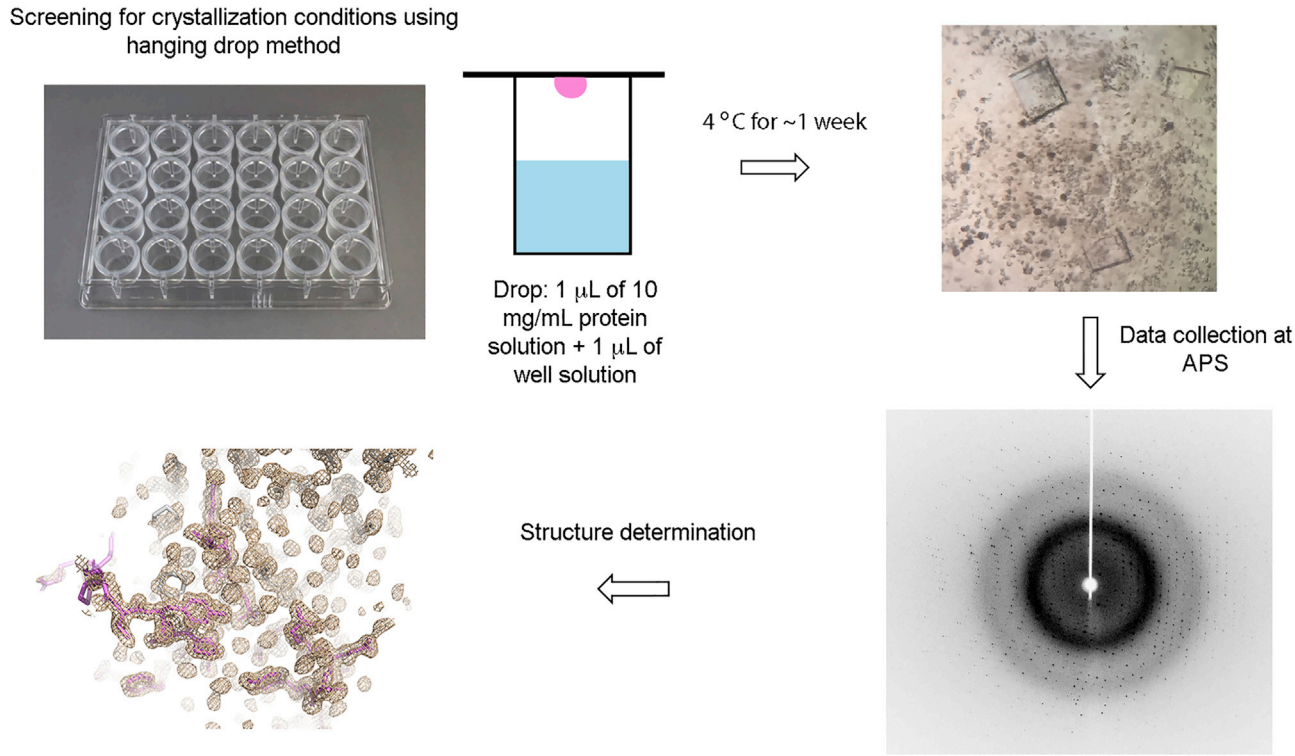

Figure 2. Structural determination of RhoA^{WT} and mutant RhoA^{G14V} in GDP-bound or GMPPNP-bound state

The crystallization process consists of a broad screening using hanging drop method, generating diffracting crystals, data collection, and structure determination.

37. Concentration of the RhoA proteins is around 8–10 mg/mL. Crystals are grown by equilibrating a mixture containing 1 μL protein solution and 1 μL reservoir solution against 700 μL reservoir solution at 4°C using the hanging drop vapor diffusion methods.
38. The RhoA crystals are obtained in 0.1 M Tris pH 8.3, 16%–20% PEG 8K, and 15% (v/v) 1, 4-dioxane. The plate crystal appears in two days, and reach the maximum size in 1–2 weeks. Troubleshooting 4
39. Loop the crystals using appropriate size loops under the optical microscope. Transfer the crystals with loops and wash them for several seconds, first in cryoprotect solution containing 0.1 M Tris pH 8.3, 15% (v/v) 1,4-dioxane, 25% (v/v) PEG 8K, and then in cryoprotect solution containing 0.1 M Tris pH 8.3, 15% (v/v) 1,4-dioxane, 30% (v/v) PEG 8K. Loop the crystals again and freeze the crystals mounted on the loops in liquid nitrogen.
40. The frozen crystals are shipped to Beamline 31D at the Advanced Photon Source (APS), Argonne National Laboratory. Use of the Lilly Research Laboratories Collaborative Access Team (LRL-CAT) beamline at Sector 31 of the Advanced Photon Source was provided by Eli Lilly Company, which operates the facility.
41. The datasets were indexed and integrated using Mosflm (Battye et al., 2011) and Scala (Evans, 2011).
42. The structure of RhoA^{WT} and RhoA^{G14V} in complex with GDP were determined by the molecular replacement method with the program Phaser (McCoy et al., 2007) using a searching model based on the structure of RhoA-GDP (PDB: 1FTN), and the structure of RhoA^{WT} in complex with GMPPNP was determined using a searching model based on the structure of constitutively active RhoA (PDB: 1KMQ).
43. Refinement was performed using Phenix (Liebschner et al., 2019) and models were built with Coot (Emsley and Cowtan, 2004) in alternative rounds.
44. Final models and scaled reflection data were deposited to the PDB (rcsb.org)

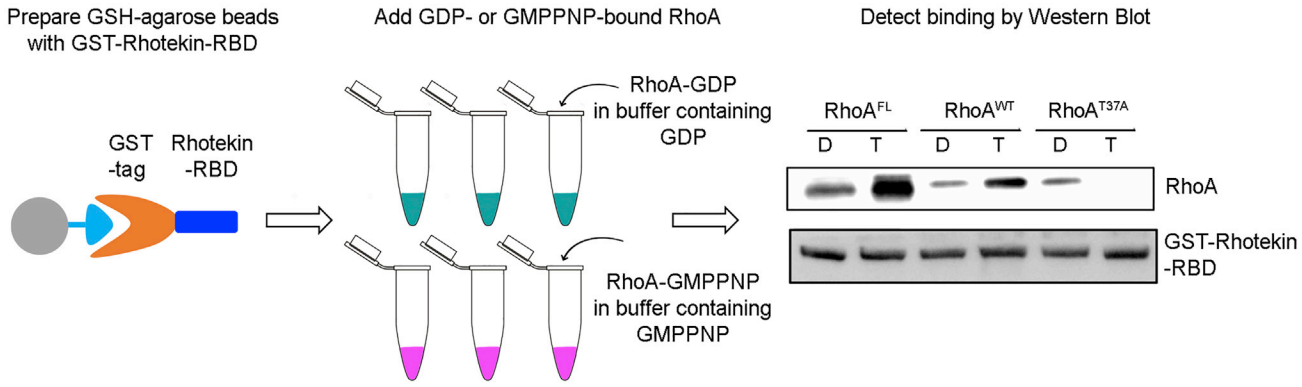

Figure 3. Procedure of GST-Rhotekin-RBD pull-down of RhoA and mutants
The pull-down reactions are done with both GDP-bound and GMPPNP-bound RhoA proteins.

Note: GDP or GMPPNP was added to the crystallization samples after purification to a final concentration of 5 mM (~10× of RhoA proteins).

GST-Rhotekin-RBD pull-down

Timing: 2–3 days

Detect the interaction between RhoA and its effector Rhotekin (Figure 3).

45. Prepare the GST pull-down buffer. Make some GST pull-down buffer supplemented with 10 μM GDP or appropriate GTP analogs. Make just enough amount as DTT is not stable and buffer should be freshly made for every experiment.
46. 10 μL GSH-agarose beads (20 μL 50% v/v slurry) are needed for each pull-down. Take the beads and wash 3 times with 1 mL GST pull-down buffer.

⚠ CRITICAL: after spinning, carefully remove the wash supernatant with a pipette at an angle to not lose any beads during the wash step.

47. Add 20 μg of the GST-Rhotekin-RBD protein to the 10 μL GSH-agarose beads. Rotate at 4°C for 30 min. Then wash with 1 mL GST pull-down buffer 3 times.

Optional: The GST-Rhotekin-RBD beads can be prepared in one tube for all pull-down reactions in one experiment, then be aliquoted to individual tubes before adding RhoA. The loading of GST-Rhotekin-RBD will be more even in this way.

48. Wash the beads in each tube with GST pull-down buffer containing either GDP or GTP analog.
49. Dilute the RhoA samples in both GDP-bound or GTP analog-bound forms to ~1 mg/mL. Carefully quantify the samples using Bradford assay.
50. Add 5 μg of each RhoA sample to the beads, rotate at 4°C for 1 h in 100 μL GST pull-down buffer containing GDP or GTP analog.

Pause point: The beads can be kept in rotation at 4°C for 12–16 h.

51. Wash the beads 2 times with 1 mL GST pull-down buffer containing GDP or GTP analog.
52. Add 20 μL 4× SDS Sample buffer, boil at 95°C for 5 min. Load all the supernatant to 15% SDS-PAGE gel and subject to subsequent anti-RhoA Western Blot analysis (RhoA antibody dilution 1:2000) to detect the RhoA and mutant proteins being pulled-down. Troubleshooting 5

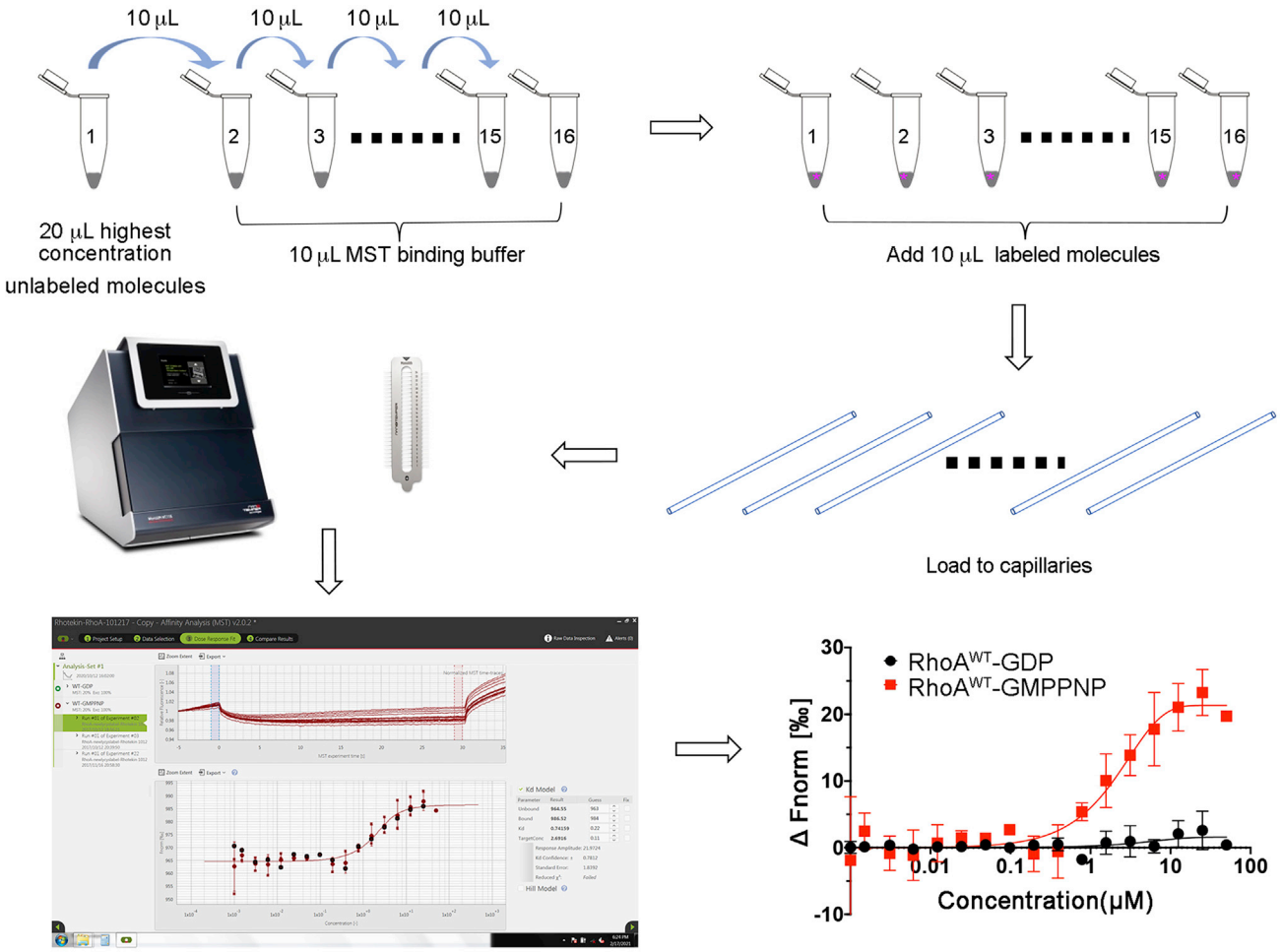

Figure 4. Characterization of the Interaction between RhoA and Rhotekin-RBD by MST

GDP-bound or GMPPNP-bound RhoA proteins are labeled and Rhotekin-RBD is titrated. Data are presented as mean ± SD.

Note: Compared with precasted 4%–15% gradient gels, 15% SDS-PAGE gels give a sharper band for RhoA protein.

MST binding assay

Timing: 2–3 days

Measure the binding affinity of the interaction between RhoA and Rhotekin-RBD using the microscale thermophoresis (MST), which is an effective technique to quantify biomolecular interactions (Jerabek-Willemsen et al., 2011) (Figure 4).

⚠ CRITICAL: Before you start, compare the Monolith systems to find the suitable one. Monolith NT.115 gives the most flexibility when it comes to labeling strategy with multiple fluorescent channels, while Monolith NT. Labelfree can measure interactions by detecting the intrinsic fluorescence of your target protein. The label-free option is ideal to characterize interactions under native conditions. However, it requires the other binding partner to have no absorbance at the UV wavelength, making it unsuitable for most protein-protein interactions. For Monolith NT.115, it is important to keep in mind that labeling may interfere with the binding and result in reduced apparent affinity.

Note: To use the NT.115 model, one binding partner will require a fluorophore label. For protein-protein interactions, NT-647 (RED) dye carrying a reactive NHS-ester group that reacts with primary amines (lysine residues) or a reactive maleimide group that reacts with cysteine residues is often used. Before the experiment, make sure that the concentration of the unlabeled partner is high enough to reach a final concentration at least 10-fold, ideally more above the expected dissociation constant (K_D). In this case, labeling RhoA protein at the cysteine residues works well.

53. Prepare labeled RhoA proteins using Monolith Protein Labeling Kit RED-MALEIMIDE (Cysteine Reactive) (NanoTemper technologies) according to the manufacturer's protocol provided in the kit (not available online). Here is a brief description:
    a. Take 20 μM, 100 μL of the RhoA protein to be labeled. Exchange the sample into the labeling buffer using column A. (Both labeling buffer and column A are provided in the kit)

Note: This step can be skipped if your sample is in a suitable buffer (such as HEPES, PBS, NaAc) with no imidazole, DTT or β-ME. Buffer exchange using column A is always recommended.

    b. Dissolve the dye in 30 μL DMSO, vortex to mix well. The resulting dye is 470 μM. Dilute the dye to 2-3 fold of the protein concentration using the labeling buffer.
    c. Mix 100 μL of protein and 100 μL of the diluted dye. Incubate for 30 min at 25°C in dark.
    d. Get rid of the extra dye using column B with MST sample storage buffer (column B is provided in the kit).
    e. Calculate the concentration of the protein and the dye using Nanodrop to measure the absorption at 280 nm for protein and 650 nm for the dye. The molar absorbance of the dye is 250,000 $M^{-1}cm^{-1}$. Troubleshooting 6
    f. Aliquot into PCR tubes, freeze in liquid nitrogen and store at −80°C.

⚠ CRITICAL: Use only highly purified protein samples for labeling.

Note: Make sure you do not have too little or too much fluorescence intensity. If the florescence intensities of the labeled protein do not match by a factor of 2-3 of the free dye at the same concentration, then the labeling efficiency is low or protein/sample concentration is not in the expected range. Too much florescence may indicate over-labeling or the presence of free dye. Free dye would strongly reduce the signal to noise ratio.

54. Assay setup pretest: before you start, make sure that you are using the optimal concentration of the labeled molecule, the correct capillary type and a buffer composition in which your sample is homogeneous.
    a. Fluorescence check
        i. Fill the labeled protein in a standard capillary and start the "capillary scan" with 50% LED power.
        ii. Make sure the reading is between 200–1500 fluorescence counts. To achieve this, sample concentration can be adjusted, or the LED power can be varied between 15% and 95%.
        iii. Choose a concentration of the labeled molecule to achieve optimal fluorescence counts.
    b. Capillary Check
        i. Prepare the labeled molecules at the concentration determined. Fill 4 standard treated capillaries, 4 hydrophilic capillaries, and 4 hydrophobic capillaries.
        ii. Pick the capillary that gives symmetrical fluorescence peak.
    c. Sample quality
        i. Prepare the labeled sample in the MST binding buffer. Fill at least 4 capillaries.
        ii. Perform the "Capillaries Scan" with the predetermined setting and measure the samples at 40% MST power. The data should have a noise of 4 units or less.
        iii. Monitor for signs of aggregation. Optimize buffer if aggregation is observed. Troubleshooting 7

55. MST binding experiment: Setting up the MST experiment with fixed concentration of the fluorescent-labeled molecule, titrated with the unlabeled molecule.
    a. Prepare 16 small micro reaction tubes. Label them from 1 to 16.
    b. Fill 20 μL of the highest concentration of the unlabeled molecule in the first tube 1.
    c. Fill 10 μL of the MST binding buffer in the tube 2–16.
    d. Transfer 10 μL of tube 1 to tube 2, mix very well by pipetting up and down several times.
    e. Repeat above step 14 times to get a serial dilution, remove 10 μL from tube 15 after mixing. Tube 16 is the buffer control.
    f. Mix 10 μL of the fluorescent-labeled molecule at double the concentrated determined with the 10 μL titrated samples, mix well by pipetting up and down several times.
    g. Incubate at 25°C for 30 min, fill the capillaries, and measure the samples with 20%, 40%, and 80% MST power.

⚠ CRITICAL: The buffer in tube 1 and the buffer in tubes 2–16 must be the same to avoid any dilution effects.

Note: Prepare at least 20 μL of sample and use the small micro reaction tubes provided in the capillary kit to minimize influence of evaporation, losing samples to the plastic reaction tubes, or pipetting errors.

56. Data analysis
    a. Start Affinity Analysis Software and load raw data.
    b. Check the "Original Fluorescence" and make sure there is no concentration dependent fluorescence changes. Typically, the intensity should no vary more than 10%. Troubleshooting 8
    c. Check the signal/noise ratio at different MST powers. Use the lowest possible MST power for the analysis, which gives a good signal to noise ratio.
    d. The standard analysis mode is "Thermophoresis" or "Thermophoresis with Jump". If more than one setting shows a result, they should yield similar affinity. The K_D can be determined using the K_D fit with the default setting.
    e. Data can be exported and loaded to GraphPad Prism for binding analysis if preferred.

Nucleotide dissociation assay

Timing: 4 h

Compare the nucleotide dissociation rate among WT and mutant RhoA proteins (Figure 5). This protocol is modified based on a previous protocol (Kanie and Jackson, 2018).

57. Load BODIPY-FL-GDP to RhoA proteins
    a. For each dissociation sample, prepare 2 μM RhoA proteins and 1 μM BODIPY-FL-GDP in the BODIPY-FL-GDP loading buffer (containing 1 mM EDTA). Make the final volume to be 95 μL. Incubate at 25°C for 2 h.
    b. Add 5 μL of 100 mM $MgCl_2$ to each sample to terminate the exchange.
    c. Add 100 μL Nucleotide dissociation assay buffer (containing 5 mM $MgCl_2$). Final concentration of RhoA is 1 μM.

⚠ CRITICAL: EDTA is required for efficient BODIPY-FL-GDP loading. After loading, excess Mg^{2+} is required to stabilize the bound nucleotide.

Note: A control sample should be prepared in parallel without the protein component.

58. Aliquot 25 μL/well of BODIPY-FL-GDP loaded RhoA samples to 384-well plate. 6 wells total for each sample.

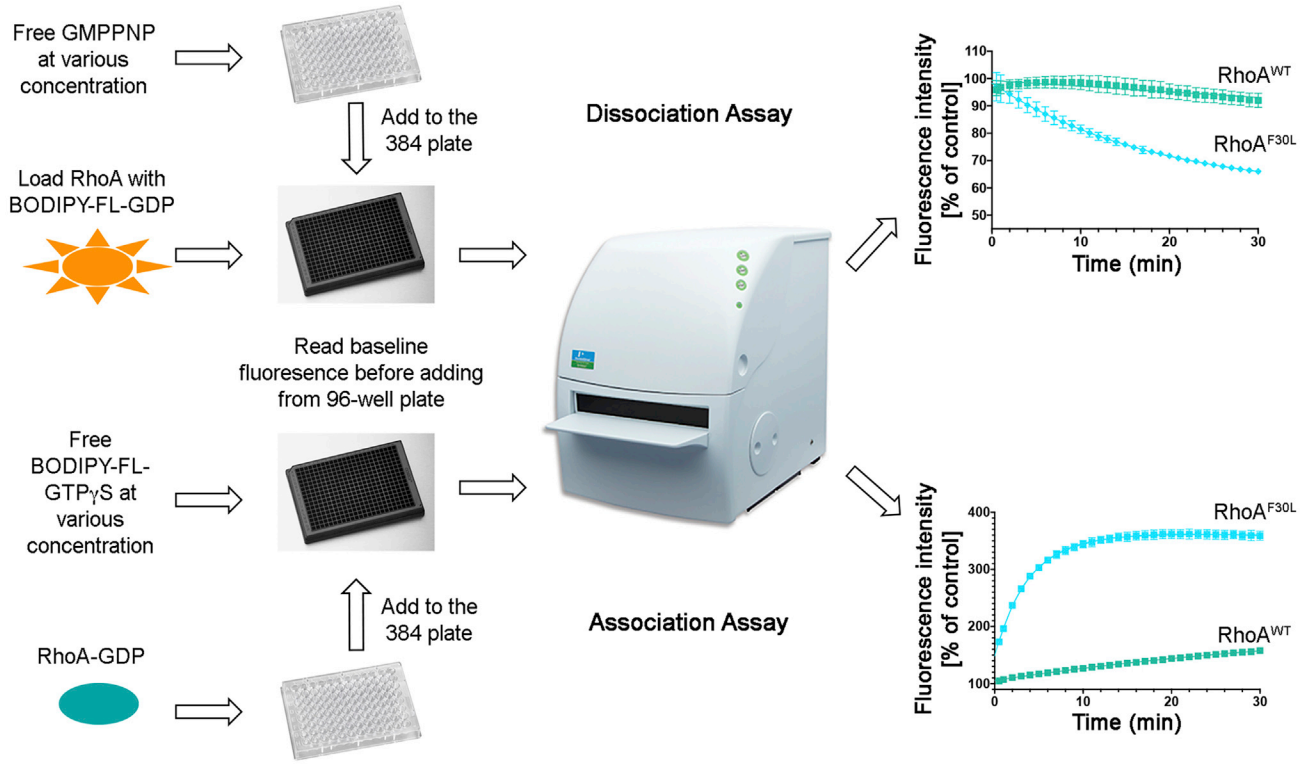

Figure 5. Procedure of the nucleotide dissociation and association assays

BODIPY-FL-GDP and BODIPY-FL-GTPγS are used for the dissociation and the association assays, respectively. These fluorescent analogs show increased fluorescence when bound to proteins. Data are presented as mean ± SD.

59. Prepare 2 μM, 5 μM, and 10 μM GMPPNP in the following GMPPNP sample in the Nucleotide dissociation assay buffer. Aliquot the GMPPNP samples to 96-well plate. The RhoA/free GMPPNP ratio is a key factor that influence the nucleotide dissociation rate. We tested 1:2, 1:5, and 1:10 RhoA : GMPPNP ratios in our study.

    Optional: GMPPNP can be prepared in a buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, and 10 mM EDTA to test the dissociation rate in the presence of EDTA. Here, EDTA will have a similar effect as RhoGEFs in accelerating the nucleotide dissociation by chelating RhoA-bound Mg^{2+}.

60. Bring the 384 plate with BODIPY-FL-GDP loaded RhoA samples and the 96-well plate with different concentration of GMPPNP to an Envision Multimode Plate Reader with excitation/emission wavelength of 485 and 535 nm. Read the 384 plate 3 times and take the average for the baseline fluorescence. The protein-free BODIPY-FL-GDP should be included as control. Troubleshooting 9
61. Add 25 μL/well of GMPPNP at varying concentrations to the BODIPY-FL-GDP loaded RhoA samples using multichannel pipette, 2 wells for every GMPPNP concentration. Read the 384 plate 3 times immediately at 1-min intervals for 30 min. The readings of 3 times are averaged and normalized as percentage of the baseline fluorescence, plotted against time.
62. Process the data using GraphPad Prism.

Nucleotide association assay

Timing: 2 h

Compare the nucleotide association rate among WT and mutant RhoA proteins (Figure 5). BODIPY-FL-GTPγS is chosen for real-time measurement of nucleotide binding (McEwen et al., 2001).

63. For each association sample, prepare 1 μM RhoA-GDP proteins in the Nucleotide association assay buffer (containing 5 mM $MgCl_2$), totally volume 200 μL.
64. Prepare 2 μM, 5 μM, and 10 μM BODIPY-FL-GTPγS in the Nucleotide association assay buffer. Aliquot 25 μL/well to 384-well plate. 2 wells for each concentration per sample.

Optional: BODIPY-FL-GTPγS can be prepared in a buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, and 10 mM EDTA to test the association rate in the presence of EDTA.

65. Add RhoA samples to 96-well plate.
66. Read the 384-well plate with free BODIPY-FL-GTPγS 3 times using plate reader. Take the average as baseline fluorescence.
67. Add 25 μL/well of the RhoA-GDP samples to the 384-well plate using multichannel pipette. Read the 384 plate 3 times immediately at 1-min intervals for 30 min. The readings of 3 times are averaged and normalized as percentage of the baseline fluorescence, plotted against time.
68. Process the data using GraphPad Prism.

EXPECTED OUTCOMES

RhoA is a well characterized member of the Rho GTPase family, which is a sub-family of the Ras superfamily of small GTPases. The conformational changes underlying activation and regulation of RhoA and related small GTPases have been an area of great interest to molecular biologists. The protocol described here can be widely applied to characterizing various small GTPases *in vitro* after minor adaptations.

In our hands, 6 L of cell culture grown in LB media yields about 30 mg of $RhoA^{FL}$ and $RhoA^{WT}$, and ~20–25 mg for $RhoA^{G14V}$ and $RhoA^{F30L}$. Low mosaic crystals of $RhoA^{WT}$ in complex with GDP and GMPPNP are expected following our protocol, diffracting to high-resolutions. We have tried to determine a $RhoA^{G14V}$-GMPPNP structure, but failed to obtain high quality diffractable crystals. There are many possibilities, one of which is related to the changed protein dynamics in the Switch regions resulted from the G14V mutation. For RhoA mutants, optimizing protein construct and crystallization condition, as well as testing different GTP analogs, are likely helpful to yield high quality crystals.

^{31}P NMR has proven useful for probing the conformational equilibrium of Ras in complex with GTP analogs. While RhoA-GMPPNP shows no obvious two states in ^{31}P spectra, similar as Rac1-GMPPCP and Cdc42-GMPPCP, RhoA-GTPγS does display two states clearly, supporting the notion that RhoA-GTP also exists in multiple conformations. RhoA-GMPPNP displays broadened resonance lines than RhoA-GDP at lower temperature, indicating possible conformational averaging. Compared to GMPPNP, GTPγS has been suggested to better mimic GTP and potentially slows down the interchange rate between the two conformational states in RhoA, allowing them to be revealed in ^{31}P NMR experiment (see more discussion in (Lin et al., 2021)). Similar strategy can be applied to study other small GTPases, or more interestingly, to study the effects of cancer-related RhoA mutants in understanding how those mutations may affect RhoA conformational equilibrium and function.

Using our protocol for the biochemistry assays, one will be able to obtain the binding affinity of RhoA with various interacting molecules, and derive the relative guanine nucleotide association and dissociation rates with or without GEFs. Such biochemistry analyses are essential to evaluate the function of RhoA and its mutants, and can be applied to most Rho and Ras-like GTPases.

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QUANTIFICATION AND STATISTICAL ANALYSIS

The crystal structures of RhoA^{WT}-GDP, RhoA^{WT}-GMPPNP, and RhoA^{G14V}-GDP were solved and refined using software listed in the key resources table. Statistics generated from X-ray crystallography data processing, refinement, and structure validation is described in (Lin et al., 2021).

MST binding assays were performed in two independent replicates, each with two independent labeling events. Similar results were obtained and one set of replicates was used for final data report. Ideally, three independent repeats are desirable. The raw data is processed first with Affinity Analysis software as described in the method details. Data is processed and normalized to ΔFnorm [‰] (10*(Fnorm(bound) - Fnorm (unbound))) for each concentration of the unlabeled molecule. The K_D values were determined by nonlinear regression fit with one-phase association model using Prism (GraphPad Software) and represent means ± SD.

For the nucleotide dissociation and association assays, the fluorescence readings are processed as percentages of the averaged baseline reading and plotted against time. Standard deviation was calculated from two or three independent experiments. Nonlinear regression is used to fit the curves and the one phase exponential model is used to obtain the pseudo first order rate constant using Prism (GraphPad Software).

LIMITATIONS

The protocols described here are for characterizations of RhoA *in vitro* in a purified system. The purification and the biochemical/biophysical methods worked well for RhoA and mutant proteins we described, including RhoA^{FL}, RhoA^{WT}, RhoA^{G14V}, and RhoA^{F30L}. However, there are some RhoA mutations that may result in unstable proteins and cannot be studied using this protocol. The examples of those mutations include RhoA^{G17V}, which is the most frequent RhoA mutation in specific subtypes of peripheral T-cell lymphomas, and RhoA^{T19N} that represents a dominant negative form. For different RhoA mutants, the purification steps need to be optimized to obtain best yield and quality. For example, RhoA^{G14V} has reduced intrinsic GTPase activity and compromised GAP-stimulated GTPase activity, therefore extra care is needed to make sure the purified protein is homogenously GDP-bound or GTP analog-bound after nucleotide exchange.

As is true for most crystal structures, non-physiological crystallization condition and crystal packing of RhoA may favor certain conformations in the defined structure. Characterization of the highly flexibly Switch regions of RhoA is challenging by structural approaches including crystallography and NMR spectroscopy. Therefore, X-ray crystal structure, NMR spectra, and molecular dynamics simulations are complementary to each other. During ^{31}P NMR spectra collection, slow hydrolyzable GTP analog GTPγS can be slowly hydrolyzed. The NMR experiment needs to be planned to minimize data collection time. As the 1D ^{31}P NMR experiment is only probing the chemical environment around the phosphorus atoms of the bound guanine nucleotide, this method is relatively crude and the spectrum changes need a careful interpretation. In addition, ^{31}P NMR experiment often fails to resolve possible conformational states, resulting in an averaged resonance. More comprehensive NMR spectroscopy approaches may be necessary to reveal more sophisticated conformational states.

Finally, the *E.coli* expressed RhoA and interactive molecules may represent only a simplified *in vitro* interacting mode. In cells, RhoA and related molecules undergo post-translational modifications that affect their intracellular localization and protein-membrane orientation in a large multimolecular complex, which enables compartmentalized signal transduction *in vivo*.

TROUBLESHOOTING

Problem 1

Low expression level for proteins (step 5).

Potential solution

Both RhoA proteins and GST-Rhotekin-RBD express better at lower temperature (18°C–20°C) with 0.2 mM IPTG for 12–16 h. The yield for cleaved RhoA is about 5–6 mg/L at 18°C–20°C, and about 3–4 mg/L at 37°C. Induction at OD_{600} around 0.8 can result in a higher level of expression for RhoA, while induction at OD_{600} around 0.6 yields a higher level of expression for GST-Rhotekin-RBD.

Problem 2

Protein not cleaved well on column (step 15).

Potential solution

GST-RhoA can be well-cleaved with about ~10 mg of thrombin at 25°C for 1 h, or 4°C for 12–16 h. If the cleavage is inefficient, check thrombin activity. On-column cleavage may not work for some GST-fusion proteins due to inaccessibility of cleavage site. In that case, elution and cleavage in solution may be needed.

Problem 3

NMR signal to noise level is too low or there are extra peaks that cannot be explained (step 32).

Potential solution

Increase the protein concentration. The protein concentration for NMR should be at least 0.4 mM. If it is hard to obtain enough protein, Shigemi NMR tubes, which require 250 μL sample, can be used instead of regular NMR tubes, which require 500 μL sample. Another option is to increase the number of scans to collect the data longer. As the ^{31}P NMR spectra are recorded at the phosphorous frequency (243 MHz) with protons decoupled, impurities usually do not contribute to the noise level. Nonetheless, highly purified protein is recommended in the NMR experiment. Spectra of free nucleotides, including GDP and GTP analogs, need to be collected in the same sample buffer to help interpret the resonance peaks of a particular protein sample. Free Pi peak and the β-phosphate resonance peak of GDP often show up on older samples, resulting from the hydrolysis of GTP analogs. The α-phosphate resonance peak of GDP is difficult to distinguish from that of GMPPNP or GTPγS when bound to RhoA, and it thereby does not show up as a separate peak most of times.

Problem 4

RhoA protein fails to be crystallized (step 38).

Potential solution

Check the purity of the protein purification. Extra GDP or GTP analogs should be added to the crystallization samples to maintain maximum nucleotide occupancy. Crystallization conditions may vary depending on the particular construct of RhoA – with or without the C-terminal, or if a particular mutation is present. Crystallization screen with matrices of the known conditions should be performed for each new construct, followed by new crystallization screens and various GTP analogs if needed.

Problem 5

GST-Rhotekin-RBD pull-down assay does not show desired band (step 52).

Potential solution

GST-Rhotekin-RBD protein needs to be freshly purified. Rhotekin-RBD is not stable and can lose its active conformation. The amounts of GST-Rhotekin-RBD and RhoA protein need to be optimized by trials such as specific RhoA band shows up in GST-Rhotekin-RBD pull-down, but not in GST pull-down.

Problem 6

Protein fluorescence signal too high or too low after labeling (step 53).

Potential solution

Make sure to follow the manufacturer's protocol and use the proper amount of protein. The protein used for labeling should be highly purified and active. Lower quality or concentration may result in loss of coupling efficiency. The dye cannot be used more than a few hours after suspension in DMSO. If the protein fluorescence signal is too high, over-labeling or free dye may be an issue. The use of lower ratio of dye to protein when labeling may help. After labeling, removing the excess dye using column B is needed as free dye could strongly reduce the signal to noise ratio.

Problem 7

MST curve is wavy or bumpy, or the repeat of the same sample gives a noise of more than 8 units (step 54).

Potential solution

The wavy or bumpy MST curve indicates sample aggregation, and the large noise indicates that the buffer composition needs to be optimized. In many cases, detergents (for example, 0.05% Tween-20) can significantly improve the homogeneity of the sample. One can also add BSA or reducing reagent to the assay buffer. Before loading the capillaries, spin the titrations at maximum speed for 5 min to get rid of any dust, air bubble, and aggregation. For samples that have the inherent property to aggregate or show only weak thermophoretic signals, enhanced gradient capillaries can be tested.

Problem 8

Concentration dependent fluorescence changes are observed (step 56).

Potential solution

Typically, the fluorescence intensity should vary randomly and no more than 10%. If there are stronger random variations, pay more attention to pipetting and use low-absorbance reaction tubes/tips. If a concentration dependent change in the fluorescence intensity, there are two possible explanations. 1) Binding of the unlabeled molecule changes the environment of the dye molecule and different labeling methods should be tested. 2) Binding of the unlabeled molecule results in unspecific adsorption or precipitation. Assay buffer should be optimized with addition of BSA/detergent, or at different pH or ionic strength.

Problem 9

Little fluorescence increase of RhoA-bound BODIPY-FL-GDP is seen compared to free BODIPY-FL-GDP (step 60).

Potential solution

This may mean that the loading of the BODIPY-FL-GDP to RhoA is not efficient. Intrinsic nucleotide exchange on RhoA is very slow and needs EDTA to chelate the Mg^{2+} cofactor in facilitating the nucleotide exchange, and in this case, loading of BODIPY-FL-GDP. After BODIPY-FL-GDP, excess Mg^{2+} is needed to terminate the exchange and stabilize the RhoA bound BODIPY-FL-GDP.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yi Zheng (yi.zheng@cchmc.org)

Materials availability

- Plasmids generated in this study are available upon request.
- This study did not generate new unique reagents.

Data and code availability

The atomic coordinates and structural factors for the crystal structures in this paper have been deposited to the Protein Data Bank, Research Collaboratory for Structure Bioinformatics (RCSB PDB, rcsb.org) with code 6V6U for $RhoA^{WT}$-GDP, 6V6M for $RhoA^{WT}$-GMPPNP, and 6V6V for $RhoA^{G14V}$-GDP.

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AUTHOR CONTRIBUTIONS

All authors contributed to writing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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